

Short communication

Gene polymorphisms of endothelial nitric oxide synthase and angiotensin-converting enzyme in patients with asthma

Background: Nitric oxide, including that produced by endothelial constitutive nitric oxide synthase (eNOS), may regulate vascular and airway tone in the lungs and may influence various aspects of airway homeostasis. Angiotensin-converting enzyme (ACE) is expressed at high levels in the lungs and plays a role in the metabolism of angiotensin II, bradykinin, and substance P, all of which are potentially involved in the pathogenesis of asthma. An insertion-deletion polymorphism of the *ACE* gene has been shown to be associated with enzyme activity levels of ACE. To examine the possible involvement of the *eNOS* and/or *ACE* genes as the genetic basis of bronchial asthma, we investigated whether there was any association between bronchial asthma and polymorphisms of the *eNOS* and/or *ACE* genes.

Methods: A total of 310 patients with bronchial asthma and 121 healthy subjects took part in this study. The *eNOS* and *ACE* genotypes were determined in all subjects by polymerase chain reaction.

Results:

- 1) The distribution of one genotype (bb) of *eNOS* was significantly higher in the asthma group than in the control population.
- 2) The *ACE* genotype distribution was not significantly different between the control and the asthma groups.
- 3) In asthmatic patients, the *ACE* and *eNOS* genotype distribution did not differ significantly among groups of patients with different severities of asthma.

Conclusions: These results suggest that polymorphisms of the *eNOS* gene, but not the *ACE* gene, may be associated with the development of asthma. However, the severity of asthma may not be influenced by polymorphisms of the *eNOS* and *ACE* genes.

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Bronchial asthma is a pulmonary disease characterized by chronic inflammation of the airways and bronchial hyperresponsiveness (1). Previous studies suggest that asthma is a multifactorial disease influenced by genetic and environmental factors (2, 3). However, the nature of the genetic factors remains unknown.

Nitric oxide (NO) plays a key role as a vasodilator, neurotransmitter, and inflammatory mediator in the airways, and it is produced in increased amounts in asthma (4, 5). NO is derived endogenously from the

amino acid L-arginine by the enzyme NO synthase, which occurs in three distinct forms (4, 5). NO produced by two types of constitutive nitric oxide synthases (cNOS), endothelial NO synthase (eNOS) or neuronal nitric oxide synthases (nNOS), is involved in physiologic regulation of airway function (4, 5). Several previous studies have suggested that cNOS is an important homeostatic regulator of microvascular permeability, neutrophil endothelial interactions, and maintenance of local perfusion, and is also a crucial defense mechanism during the onset of inflammation (6–10). Therefore, some types of gene polymorphisms of cNOS might be relevant to the pathogenesis of asthma.

Angiotensin-converting enzyme (ACE) acts as a dipeptidylcarboxypeptidase that cleaves the C terminal

Abbreviations. ACE: angiotensin-converting enzyme; cNOS: constitutive nitric oxide synthase; d-NTP: deoxynucleotide triphosphate; eNOS: endothelial constitutive nitric oxide synthase; iNOS: inducible nitric oxide synthase; nNOS: neuronal nitric oxide synthase; NO: nitric oxide; PCR: polymerase chain reaction.

dipeptide from angiotensin I to give the octapeptide angiotensin II, a potent compound that may be involved in the pathogenesis of asthma via interactions with airway smooth muscle. Previous research has also shown that ACE inactivates bradykinin and substance P, which have profound inflammatory effects in bronchial asthma (11). ACE is expressed in relatively large amounts in the lungs and plays a pivotal role in the metabolism of peptides possibly involved in the pathogenesis of asthma. An insertion/deletion (I/D) polymorphism of the *ACE* gene has been shown to correlate with circulating and cellular ACE concentrations (12–15). The DD genotype is associated with the highest plasma levels of ACE activity, while the II genotype correlates with the lowest levels (14, 15). This suggests that polymorphisms of the *ACE* gene might participate in the control of ACE activity and could be implicated as a factor in patients with asthma.

In view of the importance of these functions to the lungs, we hypothesized that abnormalities in the *ecNOS* and/or *ACE* genes may contribute to the pathogenesis of bronchial asthma. To date, however, the role of the *ecNOS* and/or *ACE* genes in asthma susceptibility and severity is inconclusive. Therefore, in this study, we investigated whether polymorphisms of the *ecNOS* and/or *ACE* genes are associated with susceptibility to bronchial asthma and/or with the clinical outcome of adult patients with bronchial asthma.

Material and methods

Subjects

A total of 310 patients with bronchial asthma (167 men and 143 women), aged 50.6 ± 20.1 years (mean $\pm$ SD), were studied. A total of 121 healthy subjects (70 men and 51 women), aged 59.8 ± 1.1 years (mean $\pm$ SD), who were free of lung and cardiovascular disease and had no clinical evidence of personal or familial atopy or asthma, were recruited as controls. All subjects were ethnic Koreans. They had no respiratory complaints, and had normal spirometric results together with normal bronchial hyperresponsiveness (PC_{20} methacholine greater than 16 mg/ml). These patients were divided into three experimental groups as follows (Table 1):

group I: normal controls ($n=121$, 59.8 ± 1.1 , 70 males/51 females)

group IIa: patients with bronchial asthma, extrinsic type ($n=236$, 48 ± 20.6 , 125 males/111 females)

group IIb: patients with bronchial asthma, intrinsic type ($n=74$, 60.9 ± 13.8 , 42 males/32 females)

Subjects with bronchial asthma were defined according to the criteria of the American Thoracic Society (16). The diagnosis of bronchial asthma was confirmed by a clear clinical history with current clinical symptoms (episodic wheezing, chest tightness, and dyspnea) and 15% or greater reversibility of FEV_1 spontaneously or after nebulized β_2 -agonist and/or a methacholine provocation test result with a PC_{20} of less than 8 mg/ml. Asthmatic subjects of the extrinsic type were defined by

- 1) a positive prick test response with extracts of one or more common aeroallergens
- 2) a positive RAST response of greater than 0.70 IU/ml to one or more of these allergens.

The severity of asthma was classified according to the guidelines of the NHLBI/WHO workshop (17).

This study was approved by the ethics committee of Chonbuk National University (South Korea), and fully informed, written consent was obtained from each subject.

Determination of *ecNOS* and *ACE* genotypes

Blood samples were collected from patients and from controls, and genomic DNA from leukocytes was prepared by standard techniques. DNA concentrations were measured by absorbance at 260 nm.

The *ACE* gene contains a polymorphism based on the presence or absence of a 287-bp nonsense DNA domain in intron 16, which results in three different genotypes: DD or II homozygotes, or ID heterozygotes. To determine the *ACE* genotype and to avoid the probability of mistyping ID heterozygotes as DD homozygotes by conventional PCR, as reported by Rigat et al. (14), we used the modified multiplexed PCR method. Three nucleotides, the sense primers 5'-CTG CAG GTG TCT GCA GCA TGT GC-3' and 5'-GAT TAC AGG CGT GAT ACA GTC ACT TTT-3', and the antisense primer, 5'-GCC ATC ACA TTC GTC AGA TCT GGT AG-3' were designed to amplify the polymorphic fragments from human genomic DNA. They allow the detection of a 237-bp fragment in the absence of the insertion, and of two fragments (525 bp and 155 bp) in the presence of the insertion. The reaction mixture (a total of 20 μ l) contained 1 U Taq DNA polymerase, 1 mM of each dNTP, 20 pmol of each primer, 40 mM KCl, 10 mM Tris-HCl (pH 8.3), 1.5 mM $MgCl_2$, 0.5 μ g/ml BSA, 12 μ l 8-MOP, and 500 ng genomic DNA. The reaction condition was achieved by an initial denaturation step at 94°C for 5 min and then repeating the following 30 cycles: denaturing at 94°C for 45 s, primer annealing at 65°C for 45 s, and primer extension at 72°C for 45 s, with a final extension at 72°C for 5 min.

Table 1. Demographics of asthmatic patients and normal control subjects

	Group I	Group II	Group IIa	Group IIb
No.	121	310	236	74
Age (years)	59.8 ± 1.1	50.6 ± 20.1	48 ± 20.6	60.9 ± 13.8
Sex (M/F)	70/51	167/143	125/111	42/32

To determine the *ecNOS* genotype, a genomic DNA fragment of intron 4 of the *ecNOS* gene was amplified by PCR. The primers used were 5'-GGA ACC TCA GCC CAG TAG TG-3' (sense) and 5'-GGC AAG TGT CAG ATA GGA TT-3' (antisense). The reaction mixture contained 100 mM Tris-HCl pH (8.3), 50 mM KCl, 1.5 mM MgCl₂, 0.25 mM of each dNTP, 100 pmol of each primer, and 1 U/μl Taq polymerase (Perkin Elmer, USA). PCR was performed with 35 cycles of 60 s at 95°C, 60 s at 56°C, and 120 s at 72°C, followed by final extension for 10 min at 72°C.

The gels were stained with ethidium bromide to display molecular weight markers and were then autoradiographed to detect alleles. When the products of different alleles varied in length, the genotypes of *ACE* and *ecNOS* genes were determined directly by electrophoresis of PCR fragments (Fig. 1).

Statistical analysis

The frequencies of genotypes among the normal controls and asthmatic patients were counted. Differences in genotype frequencies between groups were examined

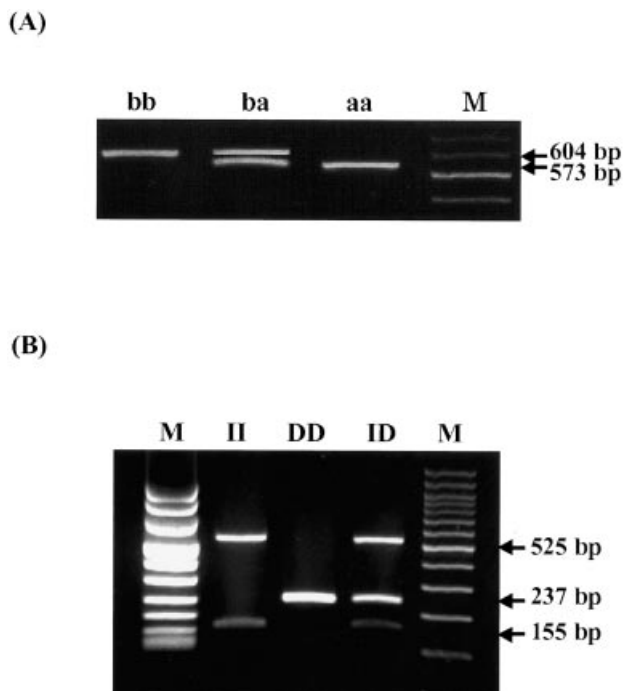


Figure 1. A) PCR products show three genotypes of *ecNOS* gene in 2% agarose gel electrophoresis. bb type shows one band at 604 bp, aa type shows one band at 573 bp, and ba type shows two bands at 604 bp and 573 bp (DNA fragment sizes, in bases, are indicated by numbers on left). B) PCR products show three genotypes of *ACE* gene in 2% agarose gel electrophoresis. DD type shows one band at 237 bp, II type shows two bands at 525 bp and 155 bp, and ID type shows three bands at 525 bp, 237 bp, and 155 bp (DNA fragment sizes, in bases, are indicated by numbers on right). M: size markers.

Table 2. Frequencies of genotypes of polymorphisms of *ecNOS* gene (A) and *ACE* gene (B)

Genotype	Group I	Group II
bb	91 (75%)	272 (88%)
ba	29 (24%)	35 (11%)
aa	1 (1%)	3 (1%)

$\chi^2 = 11.06$ (d.f. = 2, $P = 0.004$).
(B)

Genotype	Group I	Group II
II	48 (40%)	109 (35%)
ID	50 (41%)	158 (51%)
DD	23 (19%)	43 (14%)

$\chi^2 = 3.66$ (d.f. = 2, $P = 0.16$).

by the chi-square test and Fisher's exact test. Results were considered statistically significant if the P values were less than 0.05. Data are expressed as mean $\pm$ SD.

Results

In the control group, the frequencies of the bb, ba, and aa genotypes of the *ecNOS* gene were 75%, 24%, and 1%, respectively. Among the population of patients with bronchial asthma, the distributions of the bb, ba, and aa genotypes were 88%, 11%, and 1%, respectively. There were significant differences in the distribution of those genotypes in patients with asthma compared to control subjects ($\chi^2 = 11.06$; d.f. = 2, $P = 0.004$) (Table 2A).

Among the healthy control population, the frequencies of the II, ID, and DD genotypes of the *ACE* gene were 40%, 41%, and 19%, respectively. In patients with asthma, the distributions of the II, ID, and DD genotypes were 35%, 51%, and 14%, respectively. There were no significant differences between healthy subjects and patients with asthma (Table 2B).

In group IIa, the frequencies of the bb, ba, and aa genotypes of the *ecNOS* gene were 86%, 13%, and 1%, respectively. In group IIb, the frequencies of the bb, ba, and aa genotypes were 92%, 7%, and 1%, respectively. There were no significant differences between groups IIa and IIb in the distribution of genotypes in patients with asthma ($\chi^2 = 2.11$; d.f. = 2, $P = 0.35$). In group IIa, the frequencies of the II, ID, and DD genotypes of the *ACE* gene were 34%, 50%, and 16%, respectively. In group IIb, the frequencies of the II, ID, and DD genotypes were 39%, 53%, and 8%, respectively. There were no significant differences between groups IIa and IIb in the distribution of genotypes in patients with asthma ($\chi^2 = 2.83$; d.f. = 2, $P = 0.24$).

In patients with mild to moderate asthma, the frequencies of the bb, ba, and aa genotypes of the *ecNOS* gene were 85%, 14%, and 1%, respectively. In patients with severe asthma, the frequencies of the

bb, ba, and aa genotypes were 94%, 5%, and 1%, respectively. There were no significant differences between patients with mild to moderate asthma and those with severe asthma ($\chi^2=4.80$; d.f. = 2, $P=0.09$). In patients with mild to moderate asthma, the frequencies of the II, ID, and DD genotypes of the *ACE* gene were 37%, 49%, and 14%, respectively. In patients with severe asthma, the frequencies of the II, ID, and DD genotypes were 30%, 55%, and 15%, respectively. Again, there were no significant differences between patients with mild to moderate asthma and those with severe asthma ($\chi^2=1.54$; d.f. = 2, $P=0.46$).

Discussion

To our knowledge, this study is the first to report evidence for an association between polymorphisms of the *ecNOS* gene and asthma. NO was known to be a mediator of the regulation of vascular and airway tone, and to be synthesized from L-arginine by the enzyme NO synthase. NO synthase is either constitutive or can be induced by lipopolysaccharides and/or cytokines. Previous immunohistologic studies have identified three isoforms (8). The neuronal and endothelial isoforms are believed to be constitutive in nature, while the third isoform is induced by a variety of cytokines and endotoxins. NO has several effects on airway function, and there is increased production of this gas in patients with asthma, as evidenced by increased levels of NO in exhaled air from asthmatic patients (8, 9). In the lungs, NO has both beneficial and detrimental actions on the function of the airway. NO is produced by eNOS, which are tightly regulated, calcium-dependent enzymes found in specific cells, and which help regulate pulmonary blood flow and bronchodilation by smooth-muscle relaxation (5, 18). However, NO is also produced by a calcium-independent cytokine-inducible NOS (iNOS), which can be distinguished from the cNOS isoforms by its relatively large production of NO. This production can have detrimental effects and serious toxic consequences in the airway (4, 18). NO produced by eNOS may regulate platelet aggregation, leukocyte adhesion to the endothelium, monocyte chemotaxis, and the tone and mitotic activity of vascular smooth-muscle cells (4). It has been reported that the human endothelial NOS gene was assigned to the 7q35–36 region of chromosome 7 (19). Recently, Wang et al. (20) reported an association between the intron 4 variable number of tandem repeats and coronary artery disease. In addition, participation of *ecNOS* polymorphic variants in the pathogenesis of disease has been studied in hypertension and emphysema (21, 22).

In the control group, the frequencies of the bb, ba, and aa genotypes of the *ecNOS* gene were 75%, 24%, and 1%, respectively. Genotype distributions of *ecNOS* gene a/b polymorphism in our study were similar to

those of previous studies (20, 23–25). Our results show that the bb genotype of *ecNOS* was more frequent in patients with asthma than in controls. Histochemical activity and immunoreactivity of cNOS have been recently demonstrated in human lung epithelium (26, 27). Asano et al. (26) have shown that human alveolar and bronchial epithelial cells constitutively express cNOS with low basal activity regulated by Ca^{2+} perturbations; these cells also respond to IFN- γ and other synergistic combinations of cytokines with induction of an immune-type iNOS that produces high levels of NO. Shaul et al. (27) also suggested that endothelial NOS expressed constitutively in certain airway epithelial cells is at least one source of endogenous NO, which may function in a paracrine fashion to modulate bronchomotor tone. These results support the notion that NO derived from airway epithelium plays a physiologic role in normal airway luminal homeostasis, and they may provide insight into the role of NO in immune modulation and airway inflammation. Furthermore, Tsukada et al. (24) reported that *ecNOS* gene polymorphisms correlate with circulating NO concentrations, with individuals of bb genotype exhibiting the highest NO concentrations, and those of the II genotype the lowest NO concentrations. For these reasons, we suspected that the level of NO, which is produced by eNOS, might affect the development of bronchial asthma, and that polymorphisms of the *ecNOS* gene and development of asthma might be related.

In the present study, we were unable to detect any association of the *ACE* genotype with asthma, indicating that the I/D polymorphism of the *ACE* gene is not a major risk factor for the development of asthma. These data are different from those reported by Benessiano et al. (12), who found an association between the DD genotype of *ACE* and asthma. We considered several circumstances that may be responsible for these different results. First, one possible cause of the lack of an influence of the *ACE* genotype on asthma in our study, unlike the French study, might relate to ethnic differences. All subjects participating in this study were Orientals living in South Korea. The frequencies of the *ACE* genotypes in our control population were different from previously reported data obtained in other Caucasian control populations (12, 28), including those reported by Benessiano et al. (12). Second, such different results might be due to the PCR methods used for genotyping the *ACE* gene. In this study, we used the modified multiplexed PCR method because preferential amplification of the D allele may lead to overestimation of DD genotype. To clarify this matter, further study with a much larger patient population will be necessary to establish whether our results can be applied to other ethnic or national groups.

ACE inactivates bradykinin and substance P, which have inflammatory effects in asthma. The other

function of ACE is to convert angiotensin I into angiotensin II, a compound that may contribute to bronchoconstriction during acute severe asthma. These effects suggest that ACE may have either direct effects on airway smooth muscle or indirect effects through the release of other mediators of bronchoconstriction (such as endothelin) and promote the growth of cultured smooth-muscle cells (12, 28, 29). An insertion/deletion polymorphism of the *ACE* gene has been shown to be associated with differences in plasma ACE activity levels (14, 15). In view of those observations, it seems possible that a chronic increase in expression of ACE in

patients carrying the DD genotype could affect bronchial reactivity. However, our results do not support that hypothesis.

In summary, we found an association of the *ecNOS* gene polymorphism and asthma susceptibility in Orientals in South Korea. Our results represent the strongest evidence to date for a role of the *ecNOS* gene in asthma. However, further study with a much larger patient population will be necessary.

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